Prevention of *N*-Methyl-*N*-Nitrosourea-Induced Mammary Carcinogenesis in Rats by 1α-Hydroxyvitamin D_5

Rajendra Mehta, Michael Hawthorne, Laura Uselding, Dragos Albinescu, Robert Moriarty, Konstantin Christov, Rajeshwari Mehta

Background: **Although the active form of vitamin D, i.e., 1,25-dihydroxyvitamin D_3, is a potent cell-differentiating agent, its use in cancer prevention or therapy is precluded because it induces excessive blood calcium levels (hypercalcemia). However, less calcemic or noncalcemic synthetic analogues of vitamin D_3 are poorly effective against mammary carcinogenesis. We synthesized an analogue of vitamin D_5, 1α-hydroxy-24-ethylcholecalciferol (1α-hydroxyvitamin D_5), which was less calcemic than 1,25-dihydroxyvitamin D_3 and prevented the development of precancerous lesions in mammary glands. Here, we evaluate its efficacy in an experimental rat mammary carcinogenesis model.** ***Methods:*** **Sprague-Dawley rats were treated with 1α-hydroxyvitamin D_5 beginning 2 weeks before carcinogen treatment. Animals received an intravenous injection of *N*-methyl-*N*-nitrosourea at 80 days of age and continued to receive dietary 1α-hydroxyvitamin D_5 for an additional 105 days. Tumor incidence and multiplicity were determined, and plasma concentrations of calcium and phosphorus were measured. The efficacy of 1α-hydroxyvitamin D_5 at different stages of carcinogenesis was determined in mouse mammary gland organ culture. All statistical tests were two-sided.** ***Results:*** **The tumor incidence was reduced from 80% (95% confidence interval [CI] = 51.9%–95.7%) in control rats to 53.3% (95% CI = 26.6%–78.8%) and 46.6% (95% CI = 21.3%–73.4%) in rats treated with 1α-hydroxyvitamin D_5 at 25 μg/kg diet and 50 μg/kg diet, respectively. The tumor multiplicity was reduced from 1.6 tumors per rat to 1.2 (95% CI for the difference = −0.45 to 1.25; $P = .34$) and 0.8 (95% CI for the difference = 0.14–1.46; $P = .02$), respectively. There was no statistically significant increase in the plasma calcium or phosphorus concentration at either dose level. The vitamin D_5 analogue was effective during both the initiation and the promotion stages of mammary lesion formation in organ culture.** ***Conclusion:*** **Our findings indicate that 1α-hydroxyvitamin D_5 reduces the incidence of mammary carcinogenesis *in vivo*. This analogue appears to be a good candidate for further development as a chemopreventive agent. [J Natl Cancer Inst 2000;92:1836–40]**

Recently, interest has increased in less toxic cancer chemoprevention agents. This resulted from new strategies for discovering novel chemopreventive agents from natural products, the drive to make nontoxic synthetic agents, and the need to circumvent undesirable effects of chemotherapeutic agents *(1–3)*. Since vitamin D exhibits marked cell-differentiating activity, it can be used as a possible chemopreventive agent *(4,5)*. However, it induces hypercalcemia *(6)* in experimental models, precluding its use in cancer chemoprevention and therapy. Attempts have been made to synthesize a less toxic vitamin D analogue that retains its cell-differentiating properties *(7)*.

Three synthetic derivatives of vitamin D have received considerable attention: the hexafluoro analogue of vitamin D_3 (Ro24-5531) *(8)*, EB1089 *(9)*, and 22-oxa-calcitriol *(10)*. These agents either do not produce hypercalcemia or are less toxic than 1,25-dihydroxyvitamin D_3. However, none of these compounds can be tolerated at high concentrations or can reduce tumor incidence in established carcinogenesis models. Ro24-5531 can reduce the number of tumors without reducing the tumor incidence per rat in *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinogenesis when animals

Affiliations of authors: R. Mehta, M. Hawthorne, L. Uselding, K. Christov, R. Mehta (Department of Surgical Oncology, College of Medicine), D. Albinescu, R. Moriarty (Department of Chemistry, College of Arts and Sciences), University of Illinois, Chicago.

Correspondence to: Rajendra Mehta, Ph.D., Department of Surgical Oncology, College of Medicine, 840 S. Wood St. (M/C820), Chicago, IL 60612 (e-mail: raju@uic.edu).

See "Notes" following "References."

EXHIBIT B

were treated with 50 mg MNU/kg of body weight *(8)*. The tumor incidence was reduced at a lower carcinogen dose of 15 mg/kg of body weight. The high tolerated dose in that study, which did not affect body weight gain, was 2.5 nmol/kg of diet or 1.045 μg Ro24-5531/kg of diet. The other agent, EB1089, has been used in the chemotherapy protocol utilizing human breast cancer cells in athymic mouse.

We synthesized an analogue of 24-ethylvitamin D_3 (vitamin D_5 series), i.e., 1α-hydroxyvitamin D_5, and reported that it does not produce elevated blood calcium levels at an effective concentration *(11)*. An earlier toxicology study *(12)* reported that, among the six known vitamin D series (vitamin D_2 through vitamin D_6), vitamin D_5 is the least toxic and the least calcemic. Earlier, we reported that 1α-hydroxyvitamin D_5 inhibits carcinogen-induced development of preneoplastic lesions in mouse mammary gland organ culture *(11)*.

In the present study, we examined the chemopreventive nature of 1α-hydroxyvitamin D_5 in the MNU-induced rat mammary carcinogenesis model.

Materials and Methods

1α-Hydroxyvitamin D_5

The vitamin D_5 analogue, 1α-hydroxyvitamin D_5, was synthesized and characterized as described previously *(11)*. Briefly, β-sitosterol was converted to 7-hydro-β-sitosterol by allelic bromination and was further reduced to 7-dehydro-3β-sitosterol by refluxing for 6 hours with lithium aluminum hydride and tetrahydrofuran. Photolysis and thermolysis of the reaction resulted in vitamin D_5 production, and hydroxylation of vitamin D_5 by Paaren–Deluca reaction converted vitamin D_5 to 1α-hydroxyvitamin D_5. The analogue was characterized by ^1H nuclear magnetic resonance at 400 MHz mass spectrometry and infrared spectrometry. The purity of the compound was assessed by high-pressure liquid chromatography (HPLC) (Hitachi Instruments Co., Naperville, IL). The chemical properties of 1α-hydroxyvitamin D_5 were described previously *(11)*.

Dose-Selection Study

Virgin female Sprague-Dawley rats, 50 days of age, were divided into 11 groups of 10 rats each. The animals were randomly assigned to the groups according to their weights and housed in a windowless room illuminated for 14 hours daily and maintained at 22 °C. Teklad 4% diet (Teklad Co., Madison, WI) was used as a basal control diet. The experimental diet consisted of either 1,25-dihydroxyvitamin D_3 (0.8, 1.6, 3.2, 6.4, or 12.8 μg/kg diet) or 1α-hydroxyvitamin D_5 (3.2, 6.4, 12.5, 25, or 50 μg/kg diet). The rats were observed daily for any signs of lethargy or obvious visible toxic signs and were weighed weekly. At the end of 6 weeks, they were killed by CO_2 asphyxiation, and their blood was collected for calcium and phosphorus measurements.

Measurement of Calcium and Phosphorus

Plasma was separated from blood by standard procedures. The measurements of calcium and phosphorus were performed as described previously *(11)* with the use of commercially available kits (Sigma Chemical Co., St. Louis, MO).

Mammary Carcinogenesis Experiment in Rats

The carcinogenesis experiment was carried out according to the institutional guidelines as well as an approved animal protocol. Forty-five Sprague-Dawley female virgin rats, 50 days of age, were used for the study. The animals were randomly assigned to one of various groups according to their body weights at 62 days of age. The experimental diet was initiated when the rats were 65 days of age or 2 weeks before the carcinogen treatment. 1α-Hydroxyvitamin D_5 was mixed in the diet either at 25 μg/kg diet or at 50 μg/kg diet. The vitamin D analogue was dissolved in ethanol at a concentration of 2 mg/100 mL and mixed with a placebo diet containing Neobee oil (Stephan Co., Northfield, IL) and Tenox 20 (Eastman Kodak, Rochester, NY) to yield a final concentration of 1α-hydroxyvitamin D_5 of either 25 μg/kg diet or 50 μg/kg diet. The food cups containing the diet were replaced with fresh diet twice weekly. We evaluated the integrity of vitamin D_5 in the diet by extracting the diet with methanol and injecting a reconstituted extract on an HPLC C-18 reversed-phase analytical column. The steroid was eluted with the mobile phase of acetonitrile–methanol–water (52 : 30 : 18, vol/vol) with the retention time of 33–34 minutes as described previously *(11)*. The vitamin D_5 analogue was found to be retained in the diet with 90% purity between the food cup changes.

For the induction of mammary tumors, the rats were given a single intravenous injection of 50 mg of acidified (pH 5.0) MNU per kilogram of body weight at 80 days of age as described previously *(13,14)*. If MNU is administered on day 50, it induces a nearly 100% incidence of mammary adenocarcinoma in rats at approximately 100 days after carcinogen treatment *(14)*. However, the incidence is lowered if the carcinogen is injected later in life *(14)*. The rats were monitored for their general state of health, weighed once a week throughout the experiment, and palpated for tumors once a week. They were killed at 110 days after carcinogen treatment (at 190 days of age) by CO_2 asphyxiation. Their blood was collected for calcium and phosphorus measurements. The different treatment groups were compared with the untreated control group at the end of the study. Small portions of mammary tumors, mammary glands, livers, and kidneys were fixed in formalin for histopathologic evaluation, and the rest of the tissues were frozen in liquid nitrogen for biochemical analysis.

Initiation and Promotion of Carcinogenesis in Mouse Mammary Organ Culture

The effects of 1α-hydroxyvitamin D_5 at different stages of carcinogenesis can best be determined in the same *in vivo* model. However, as a preliminary experiment, the stage-specific effects of 1α-hydroxyvitamin D_5 were determined by using a mouse mammary gland organ culture model. Mammary gland organ culture has been used successfully for evaluating the efficacy of potential chemopreventive agents before their evaluation *in vivo (11)*. In the previous study *(11)*, 1α-hydroxyvitamin D_5 was found to be effective in inhibiting 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary gland alveolar tissue lesions *in vitro*.

In the present study, we selectively examined the efficacy of 1α-hydroxyvitamin D_5 during the initiation and promotion phases of mammary gland alveolar tissue lesion formation. The mammary gland organ culture procedure for inducing mammary alveolar lesions in response to DMBA has been described in detail previously *(16)*. Briefly, 40 thoracic mammary glands from estrogen- and progesterone-pretreated BALB/c mice were divided into four groups of 10 glands each. All of the glands were incubated with growth-promoting hormones (insulin and prolactin [5 μg/mL each], aldosterone [1 μg/mL], and hydrocortisone [1 μg/mL]) for the initial 10 days of culture. The glands were treated with 2 μg/mL of DMBA for 24 hours on day 3 of a 24-day culture. The control group did not receive any vitamin D, whereas the other groups of glands received 1α-hydroxyvitamin D_5 at different times: 1) continuously during the first 10 days (initiation and growth-promoting phase), 2) only before and during the carcinogen treatment (days 0–4 of culture, initiation phase), or 3) only after the withdrawal of the carcinogen (days 4–10). After 10 days of culture, the glands were incubated with insulin (5 μg/mL) alone for an additional 14 days. At the end of the 24-day culture period, the glands were fixed in formalin and stained with alum carmine for identification of alveolar lesions.

Statistical Analysis

The tumor incidence was evaluated by survival functions with the use of the log-rank analysis. The multiplicity results were analyzed by Student's *t* test, and the two-tailed statistical significance was determined. The concentrations of calcium and phosphorus were compared by Student's *t* test for equality of means. The effects of vitamin D_5 on the initiation and promotion phases of DMBA-induced mammary lesions in organ culture were evaluated by χ^2 analysis. The statistical calculations were carried out by SPSS software packages (SPSS Inc., Chicago, IL). All statistical tests were two-sided.

Results

Dose-Selection Study

Before a carcinogenesis experiment was conducted, the maximum tolerated dose (MTD) was determined for 1α-hydroxyvitamin D_5 in comparison to 1,25-dihydroxyvitamin D_3. The results showed that body weight gain was not reduced with up to 6.4 μg of 1,25-dihydroxyvitamin D_3/kg diet. However, reduction in body weight gain was observed at 12.8 μg/kg diet. The terminal body weight in control rats ranged from 238 to 279 g as compared with 173–243 g in 1,25-dihydroxyvitamin D_3-treated rats (data not shown). These results indicate

that the MTD for 1,25-dihydroxyvitamin D_3 is less than 12.8 μg/kg diet. For 1α-hydroxyvitamin D_5, no body weight loss was observed at any dose level. At the highest concentration tested at 50 μg of 1α-hydroxyvitamin D_5/kg diet, there was a 113% gain in the body weight as compared with the control animals. At reduced concentrations, there were weight gains of 98%–99% of the control body weight. From this experiment, we selected the concentrations of 25 μg and 50 μg of 1α-hydroxyvitamin D_5/kg of diet for the carcinogenesis study.

Carcinogenesis Study

As described in the "Materials and Methods" section, all animals were given at 80 days of age an intravenous injection of 50 mg of acidified MNU (pH 5.0)/kg body weight in the jugular vein while they were under light anesthesia. The animals received 1α-hydroxyvitamin D_5 at either 25 or 50 μg/kg basal diet beginning 2 weeks before carcinogen treatment. The control group received the basal diet containing the vehicle alone.

As shown in Fig. 1, A, the tumor incidence in the control rats was 80%; 12 (80%) of 15 rats had palpable tumors (95% confidence interval = 51.9%–95.7%). 1α-Hydroxyvitamin D_5 inhibited the incidence by 33% in the group given 25 μg/kg diet (tumors in eight of 15 rats; incidence = 53.3%) and by 42% in the group given 50 μg/kg diet (tumors in seven of 15 rats; incidence = 46.6%). The reduced incidence in the high-dose group was statistically significant compared with the control group (χ^2 test; P = .03). Moreover, the disease-free survival was determined with the use of the Kaplan–Meier test for the equality of survival distribution for treatment. We used SPSS statistical software for this analysis. Results showed that the group treated with the high dose was statistically significantly different for tumor incidence from the control group (log-rank analysis; P = .029). The difference in the tumor incidence between the control and low-dose groups was not statistically significant (P = .12). When all three groups were compared, the log-rank analysis showed P = .0495.

The tumor multiplicity also was reduced from 1.6 tumors per rat in the control group to 1.2 in rats given the low dose (95% CI of the difference = −0.45 to 1.25; P = .34) and 0.8 in rats given the high dose (95% CI of the difference = 0.14–1.46; P = .02) (Fig. 1, B). Again, the difference in tumor multiplicity between the control and high-dose groups showed statistical significance (two-tailed Student's t test; P = .02), whereas the difference between the number of tumors per rat in the control and low-dose vitamin D_5 groups did not show statistical significance. Also, in the control group, the first palpable tumor appeared 40 days after carcinogen treatment. The occurrence of palpable tumor in the low-dose group was not notably delayed; four tumors appeared at 48 days after the carcinogen treatment or 63 days after the initiation of the vitamin D_5-containing diet. In the high-dose group, the first tumor appeared 57 days after carcinogen treatment or 72 days after dietary modulation. Thus, latency increased 17 days between the control and high-dose groups of animals.

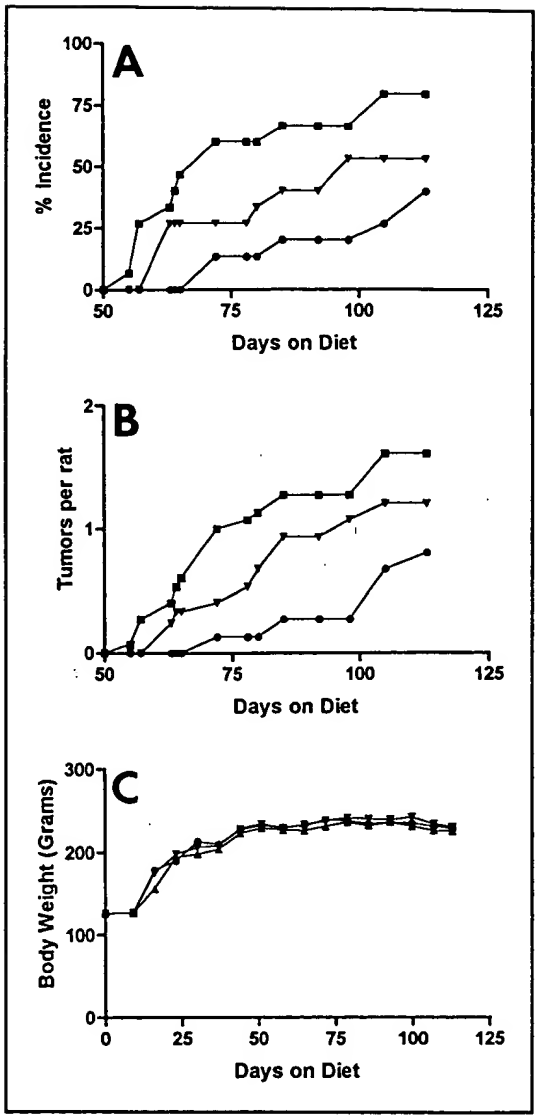

Fig. 1. Effects of 1α-hydroxyvitamin D_5 on *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinogenesis. Sprague-Dawley female rats were given an intravenous injection of 50 mg of MNU/kg body weight at 80 days of age. The rats were treated with basal diet (■) or a diet supplemented with either 25 μg of 1α-hydroxyvitamin D_5/kg of diet (▼) or 50 μg of 1α-hydroxyvitamin D_5/kg of diet (●) beginning at 65 days of age, and the treatment was continued until the end of the study. The animals were palpated for the presence of tumor and weighed once weekly. They were killed 120 days after the beginning of the diet. The effects of 1α-hydroxyvitamin D_5 were evaluated on tumor incidence (**A**) and multiplicity (**B**) as well as on body weight (**C**). **Panel A:** The tumor incidence measured at the end of the study was 80% in the control group (12 of 15 animals), with 95% confidence interval (CI) = 51.9%–95.7%; in contrast, for the rats treated with the low dose of 1α-hydroxyvitamin D_5, the final incidence was 53.3% (eight of 15 animals), with 95% CI = 26.6%–78.8%. In the group treated with the high dose of 1α-hydroxyvitamin D_5, the incidence was 46.6% (seven of 15 animals), with 95% CI = 21.3%–73.4%. **Panel B:** There was a reduction in the tumor multiplicity from 1.6 tumors per animal in the control group to 1.2 tumors per animal in the low-dose group when measured at the end of the study, with 95% CI for the difference = −0.45 to 1.25 (P = .34). In the high-dose group, the tumor multiplicity was reduced to 0.8 tumor per animal, with 95% CI for the difference = 0.14–1.46 (P = .02). **Panel C:** The body weight in the control group measured at the end of the study was not statistically different from that in the high-dose group (P = .135), with 95% CI for the difference between the two groups = −5.78 to 1.28. Similarly, the difference in the body weight between the control group and the low-dose group was not statistically different (P = .066), with 95% CI for the difference = −0.25 to 4.25.

The body weights of the animals did not differ throughout the experiment. The final average body weight of the control rats was 228 g, compared with 230 and 226 g, respectively, in the groups treated with 1α-hydroxyvitamin D_5 at 25 μg/kg diet and 50 μg/kg diet (Fig. 1, C).

These results indicate the chemopreventive nature of 1α-hydroxyvitamin D_5 at a nontoxic concentration of 50 μg/kg diet. The lower dose resulted in intermediate results.

Stage-Specific Effects in Mammary Gland Organ Culture

A preliminary experiment was performed with 10 mammary glands per group to determine if 1α-hydroxyvitamin

D_5 has stage-specific inhibition. The control glands treated with DMBA exhibited a 90% (nine of 10) incidence of alveolar lesion formation. Incubating the glands with 1 μM 1α-hydroxyvitamin D_5 for the first 10 days of a 24-day culture period resulted in a 66.7% inhibition, with only three of 10 glands containing mammary lesions (two-tailed χ^2 test; $P = .006$). This result was consistent with our previous finding *(11)*. When the exposure of the glands to the vitamin D_5 analogue was restricted to 0–4 days or 4–10 days, either treatment offered little selective influence on the overall inhibitory activity. When the glands were exposed only during 0–4 days, there was a 67% reduction in the development of mammary lesions compared with a 56% (four of 10 glands with lesions) inhibition of lesion formation when the glands were exposed during days 4–10 (promotion phase). Thus, inhibition by 1α-hydroxyvitamin D_5 during both phases was significantly different from the control ($P = .019$). However, there was no difference between the effect of the presence of the vitamin D_5 analogue either during 0–4 days or 4–10 days in culture. These results suggest that vitamin D_5 could provide a preventive effect during both initiation and promotion of the lesion development.

Toxicity

Since vitamin D-associated hypercalcemia is a well-established toxicity of 1,25-dihydroxyvitamin D_3, we measured calcium and phosphorus concentrations in the blood collected from all animals at the conclusion of the experiment. As shown in Table 1, while the concentration of phosphorus among the groups was not affected, calcium concentration increased slightly (but statistically nonsignificantly) from (mean ± standard deviation) 9.41 ± 1.19 mg/dL in the control group to 10.4 ± 1.74 mg/dL in the high-dose group ($P = .096$; 95% CI of the difference = −2.217 to 0.196). The calcium concentration ranged from 7.03 mg/dL to 11.22 mg/dL in the control group compared with 7.64 mg/dL to 15.20 mg/dL in the high-dose group. The calcium concentration in the low-dose group was 8.54 ± 1.10 mg/dL, which was also not statistically different from that of the control group ($P = .062$; the 95% CI of the difference = −0.048 to 1.789). These results confirm our previous finding that 1α-hydroxyvitamin D_5 at up to 50 μg/kg diet does not induce hypercalcemia in animals.

The effects of 1α-hydroxyvitamin D_5 on liver and kidney pathology were determined, since stone formation is often a toxic function of hypercalcemic vitamin D analogues. Histopathologic evaluations indicated normal liver and kidney structures without any pathologic abnormalities or deposition of calcium granules (data not shown). The histopathologic characteristics of the mammary gland, mammary tumors, and liver were normal.

Discussion

The active metabolite of vitamin D_3, i.e., 1,25-dihydroxyvitamin D_3, has been shown to be an excellent antiproliferative agent against various cell types *(4,5)*, but it has not progressed to clinical use because of its undesired side effects, e.g., increased plasma calcium levels and kidney toxicity *(6)*. To decrease the toxicity of vitamin D compounds without affecting their chemopreventive effects, several hundred analogues have been synthesized. Only a few analogues have shown promise, including the hexafluoro analogue of vitamin D_3 (Ro24-5531) *(8)*, 22-oxa-calcitriol *(10)*, 1α-hydroxyvitamin D_2 *(17)*, and EB1089 *(9)*. Only Ro24-5531 and 22-oxa-calcitriol have been investigated for their efficacy against carcinogen-induced mammary carcinogenesis in rats. The results indicated that 80% of the MTD of Ro24-5531 reduced tumor multiplicity and did not have any preventive effects on the incidence of mammary tumors in rats treated with a high dose of MNU. Thus, the possibility of developing yet another vitamin D analogue that would be efficacious against mammary carcinogenesis and would be tolerated at a higher concentration by rats is still desirable.

Vitamin D has been divided into six structural classes, classes D_2 through D_7. Earlier, Napoli et al. *(12)* reported that, among all of the structural varieties of vitamin D, vitamin D_5 was least toxic. Three years ago, we synthesized a novel analogue of vitamin D, 1α-hydroxyvitamin D_5, which apparently was less calcemic than 1,25-dihydroxyvitamin D_3 and substantially inhibited the development of carcinogen-induced mammary lesions in mouse mammary gland organ culture *(11)*. We also showed that the effect might be vitamin D receptor mediated, since the vitamin D_5 analogue can associate with the vitamin D receptor and facilitate vitamin D receptor–vitamin D response element interactions *(18)*. Therefore, the investigation described in this report to determine how much 1α-hydroxyvitamin D_5 can be tolerated and whether it is efficacious against MNU-induced mammary carcinogenesis is a logical extension of the experiments reported earlier *(11)*.

Earlier studies on the experimental rat mammary carcinogenesis model using the vitamin D analogue were performed with Ro24-5531. The MTD for this noncalcemic hexafluoro analogue of vitamin D_3 was found to be 1.5 μg/kg diet *(8)*. In comparison, 1α-hydroxyvitamin D_5 did not show any toxicity at the maximum concentration of 50 μg/kg diet used in dose-selection studies. In the carcinogen-

Table 1. Effects of 1α-hydroxyvitamin D_5 [1α(OH)D_5] on mammary carcinogenesis

Group No.	Treatment	No. of rats with tumors/ total No. (%)	Multiplicity	Tumor latency, days after carcinogen	Terminal body weight, g	Plasma calcium, mg/dL ± standard deviation	Plasma phosphorus,* mg/dL ± standard deviation
1	Control	12/15 (80)	1.6	40	228	9.41 ± 1.19	4.68 ± 0.14
2	1α(OH)D_5, 25 μg/kg diet	8/15 (53.3)	1.2†	48	230	8.54 ± 1.10‡	4.67 ± 0.26
3	1α(OH)D_5, 50 μg/kg diet	7/15 (46.7)§	0.8‖	57	226	10.4 ± 1.74¶	4.41 ± 0.15

*Both treatment groups statistically not different from the control group ($P = .671$; 95% confidence interval [CI] = 0.160–0.376 for the high-dose group).

†Statistically not different from the control group (Student's t test [two-tailed] $P = .34$).

‡Statistically not different from the control group (Student's t test [two-tailed] for equality of means, $P = .062$; 95% CI = −0.048 to 1.789).

§Significantly different from control group (chi-square $P<.029$). The disease-free survival curves were compared. The treatment group was significantly different from the control group (log-rank $P = .0164$).

‖Statistically different from the control group (Student's t test [two-tailed] $P = .02$).

¶Statistically not different from the control group (Student's t test [two-tailed] for equality of means, $P = .096$; 95% CI = −2.217 to 0.196).

esis experiment, the calcium level showed a possible increasing trend (in three of 15 animals) at 50 μg/kg diet. These results indicate that 50 μg/kg may be the true MTD for this agent.

Traditionally, chemopreventive agents selected for clinical trials (such as *N*-[4-hydroxyphenyl]retinamide) and several other established chemopreventive agents reduce tumor multiplicity but do not reduce the number of animals bearing tumors (tumor incidence) *(13,14)*. In contrast, 1α-hydroxyvitamin D_5 reduced tumor incidence in a dose-dependent manner. The lower dose reduced tumor incidence from 80% to 53.3% (33.3% inhibition), and the higher dose reduced tumor incidence to 46.6% (40% reduction). However, there appears to be a trend of increasing incidence of tumor formation at the higher dose, whereas a cytostatic response is observed at the lower dose. Tumor multiplicity in the control group receiving the basal diet in the present experiment was found to be 1.6 tumors per rat compared with four to six tumors per rat in other reported experiments from our laboratory and other laboratories *(13–15)*. The reason for this reduced tumor multiplicity is that the animals were treated with MNU at 80 days of age rather than at 50 days of age, as was done in the previous studies. Younger rats normally respond to carcinogen(s) more effectively than older rats *(15)*. However, we considered that keeping the physiologic estrogen level similar for the animals at the times that the vitamin D treatment was begun might be important. Therefore, rather than initiating the dietary treatment with 1α-hydroxyvitamin D_5 when the animals were at the premature age of 35 days, we initiated it when they were 65 days of age.

The current carcinogenesis study did not allow us to determine whether 1α-hydroxyvitamin D_5 is active during the initiation phase or the promotion phase of disease progression, since the chemopreventive agent is present from 2 weeks before carcinogen treatment to the end of the experiment. To determine the stage specificity of precancerous lesion formation, we evaluated the effect of the vitamin D analogue on both the initiation and the promotion phases in mouse mammary gland organ cultures. The effectiveness of the vitamin D_5 analogue on suppressing the development of mammary alveolar lesions did not show any selectivity. The extent of inhibition was consistent, regardless of whether the compound was present in the medium before or after the carcinogen treatment.

The main concern for vitamin D toxicity is the calcemic activity. Ideally, one desires a noncalcemic, efficacious compound. Earlier, we examined the calcemic nature of 1α-hydroxyvitamin D_5 in vitamin D-deficient rats and showed that, at equimolar concentrations, the D_5 analogue is dramatically less calcemic than 1,25-dihydroxyvitamin D_3 *(11)*. In the present experiment, we report that the plasma calcium concentration was not elevated at 25 μg/kg diet but was slightly elevated (but statistically not significantly) at 50 μg/kg diet (from 9.41 mg/dL in control rats to 10.4 mg/dL in the rats given the 50 μg/kg diet). These results suggest that we are working with a compound that has retained the classical vitamin D-like activity yet can be tolerated at an efficacious dose without elevating plasma calcium levels. Secondly, nephrotoxicity is often associated with vitamin D toxicity. We show here that the kidney histopathology did not differ when studied at the termination of 110 days of dietary treatment with the vitamin D analogue. These results support the findings of our previous studies anticipating the chemoprevention of primary mammary tumor development by 1α-hydroxyvitamin D_5. Our results clearly suggest that 1α-hydroxyvitamin D_5 is a potent chemopreventive agent that can be further developed as a possible chemopreventive agent for human use.

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Notes

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